INHIBITORS OF HIV REPLICATION AND METHOD OF TREATMENT OF HIV INFECTIONS

FIELD OF THE INVENTION

The invention is drawn to a novel class of drugs directed against HIV, inhibiting the dimerization of reverse transcriptase of the virus, and to a method of treatment of HIV infections, in particular multidrug-resistant HIV infections.

BACKGROUND OF THE INVENTION

In the last decade, a number of molecules have become available for the treatment of HIV-infected individuals. Therapeutic regimens based on the combination of reverse transcriptase inhibitors and protease inhibitors have been shown to reduce plasma HIV-1 RNA to undetectable levels in patients, increase CD4 cell counts and delay progression toward AIDS.

HIV reverse transcriptase (RT) inhibitors that target the polymerase activity of RT, can be subdivided into two classes of potent agents: nucleosides that terminate viral DNA synthesis, such as zidovudine (AZT), dideoxyinosine (ddI) and dideoxycytidine (ddC), and nonnucleoside analogs that bind to a hydrophobic cavity adjacent to the polymerase active site such as nevapirine (1). However, these agents present several limitations, including toxicity which sometimes requires patient's treatment to be suspended (2), and the emergence of resistant strains which are generated through the exceptionally high rate of mutagenesis of RNA viruses (3-6). For example, resistance to zidovudine is conferred by amino acid changes that appear in an orderly fashion : a K70R mutation first, followed by T125F/Y, M41L, D67N, and K219Q mutations (7,8). Similarly, other mutations correlate with resistant phenotype to other RT inhibitors (9). Thus, the development of novel compounds that are active against multidrug-resistant HIV variants is urgently needed.

An interesting feature of HIV-1 RT is that the dimeric form of the enzyme consisting of two polypeptides p66 and p51, is absolutely required for its catalytic activities (10). Based on the x-ray crystallographic structure of HIV-1 RT, it was previously demonstrated that the first interaction between p66 and p51 occurs in a Tryptophan (Trp)-rich hydrophobic cluster located in the connection subdomain of the two subunits and is followed by a conformational

change involving the thumb and the finger subdomains of p51 as well as the RNase-H and the palm subdomains of p66 (11).

SUMMARY OF THE INVENTION

The present invention is based on the concept that the dimerization process of RT could be an interesting target for AIDS chemotherapy, and on the description of new inhibitors of HIV replication, based on the inhibition of RT dimerization. These inhibitors comprise peptides that will interact with the conserved motif necessary for dimerization of the p51 and p66 subunit of the HIV-RT.

Based on the concept that a small ligand of the connection subdomains could inhibit RT dimerization, a short 10-residue synthetic peptide (p7) derived from the Trp-rich cluster at the interface of the connection subdomains of the p66 and p51 (KETWETWWTE; residues 395-404 of HIV-1 BH_{10} RT, SEQ ID N° 1) was designed. This peptide p7 is a powerful inhibitor of HIV-1 RT dimerization in vitro and abolishes the production of viral particles in HIV-1BRU-infected cultured CEM cells at a concentration of 10^{-7} M, or 10^{-8} M when complexed with the carrier peptidyl system MPG previously shown to improves the delivery of molecules into cells (14 and 15, both incorporated herein by reference in their totality). Interestingly, p7 does not exhibit any toxicity in CEM cells at concentrations below 10^{-5} M. These encouraging studies prompted to pursue the characterization of this compound as a model for potential new antiviral drugs.

The present application demonstrates the potency of the MPG/p7 complex in the abolition of the production of HIV-1 and HIV-2 viruses and demonstrates that MPG/p7 is also a potent inhibitor of drug resistant adapted HIV-1 strains.

DESCRIPTION OF THE FIGURES

Figure 1: Effect of different concentrations of MPG/p7 on HIV-1 and HIV-2 in CEM cell cultures. CEM cells exposed to 100 µl of viral suspensions containing 1000 X 50% tissue culture infective dose ($TCID_{50}$)/ml of HIV-1 BRU (left panels) or HIV-2 ROD (right panels). ***a***, HIV-infected cells were cultured in medium alone (white diamonds) or medium supplemented with MPG/p7 at 10^{-7} M (black circles), 10^{-8} M, (black up triangles), 10^{-9} M (black diamonds), and 10^{-10} M (black squares). ***b***, As controls, HIV-infected cells were treated with either azidothymidine (AZT: 10^{-5} M) (white squares) or peptide MPG/p237 at 10^{-6} M (black down triangles). Viral production was monitored by measuring RT activity

twice a week post infection. Culture supernatants from virus-free CEM were tested as a control (white circles, Fig. 1*b*).

Figure 2: Effect of MPG/p7 on replication of different subtypes of HIV-1 and HIV-2 studied using MAGIC5 cells. MAGIC-5 cells were incubated with 50 µl of stock HIV preparation corresponding to 1000 x $TCID_{50}$/ml. ***a***, HIV-1 BRU; ***b***, HIV-1 RF; ***c***, HIV-1 SF2 (Figure 2.A); ***d***, HIV-1 NDK; ***e***, HIV-1 ELI; ***f***, HIV-2 ROD; ***g***, HIV-2 EHO (Figure 2.B) in medium alone (lane 2), medium supplemented with AZT (10^{-5} M) (lane 3), MPG/p7 at 10^{-7} and 10^{-8} M (lane 4 and 5 respectively), or MPG/p237 at 10^{-6} M (lane 6) additive. After 3 days in culture, β-gal activity was evaluated in cell lysates by measuring absorbance at 410 nm. β-gal activity in uninfected MAGIC5 cells was measured as control (lane 1). All results have been normalized with respect to β-gal activity induced by each virus (100% induction). The calculated values represent means of duplicate. Each figure is representative of at least three independent experiments.

Figure 3: Effect of MPG/p7 on replication of reference escape variant viruses. MAGIC-5 cells were incubated with 50 µl of stock HIV preparation corresponding to 1000 x $TCID_{50}$/ml of ***a***, HIV-1 BRU; ***b***, HIV-1 RTMF; ***c***, HIV-1 RTMC ; ***d***, HIV-1 74V (Figure 3.A); ***e***, HIV-1 N119; ***f***, HIV-1 RTMDR1 (Figure 3.B), in medium alone (lane 2) or medium supplemented with AZT at10^{-5} M, 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, and 10^{-10} M (lanes 3 to 8 respectively), MPG/p7 at 10^{-8} and 10^{-7} M (lanes 9 and 10), or MPG/p237 at 10^{-6} M (lane 11) additive. β-gal activity was evaluated on day 3 after virus exposure. β-gal activity in uninfected MAGIC5 cells was measured as control (lane 1). All results have been normalized (see legend of Fig. 2). The calculated values represent means of duplicate. Each figure is representative of at least three independent experiments.

Figure 4: Effect of MPG/p7, peptide p7+ and peptide p7++ on replication of reference viruses. MAGIC-5 cells were incubated with 50 µl of stock HIV preparation corresponding to 1000 x $TCID_{50}$/ml of ***a***, HIV-1 BRU; ***b***, HIV-2 ROD (Figure 4.A); ***c***, HIV-1 RTMF ; ***d***, HIV-1 RTMC (Figure 4. B); ***e***, HIV-1 74V; ***f***, HIV-1 RTMDR1 (Figure 4.C). NI: non infected; BRU: infected by virus BRU without inhibitor (or other viruses in other panels); AZT: medium supplemented with AZT at10^{-5} M; MPG/p7: medium supplemented with MPG/p7 at the indicated concentration; p7+: medium supplemented with p7+ at the indicated concentration; p7++: medium supplemented with p7++ at the indicated concentration; 237+: medium supplemented with MPG/237 at the indicated concentration; β-gal activity was evaluated on day 3 after virus exposure. All results have been normalized (see legend of Fig.

2). The calculated values represent means of duplicate. Each figure is representative of at least three independent experiments.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is therefore drawn to an inhibitor of HIV replication, comprising a peptide or analog comprising a decapeptide, said decapeptide containing (from the N-terminus to the C-terminus) a basic amino acid in position 1, an acidic amino acid in positions 2 and 5, and a tryptophan in positions 4, 7, and 8.

By inhibition of HIV replication, it is meant an inhibition of the production of viral particles from infected cells. This inhibition may be measured by different means, in particular as described in Morris *et al.* (15, which is incorporated herein by reference in totality).

In particular, an inhibitor of HIV replication according to the present invention reduces the β-galactosidase activity measured from MAGIC-5 cells by at least 50 %, more preferably 70 %, the most preferably 90 % in the conditions reported in the examples of the present invention.

In a preferred embodiment, the basic amino acid in position 1 is arginine, or more preferably lysine.

In another embodiment, the acidic amino acid in position 2 is glutamate (also named glutamic acid).

In another embodiment, the acidic amino acid in position 5 is glutamate.

In another embodiment, the amino acid in position 3 is chosen in the group consisting of threonine, isoleucine and valine, and is preferably threonine.

In another embodiment, the amino acid in position 6 is chosen in the group consisting of threonine, alanine and glutamine and is preferably threonine or alanine.

In another embodiment, the amino acid in position 9 is chosen in the group consisting of threonine, alanine, valine, isoleucine, methionine, and aspartate (also named aspartic acid), and is preferably threonine.

In another embodiment, the amino acid in position 10 is chosen in the group consisting of glutamate, aspartate and asparagine, and is preferably glutamate or aspartate, more preferably glutamate.

In a preferred embodiment, the inhibitor of the invention comprises a decapeptide containing (from the N-terminus to the C-terminus) a basic amino acid in position 1, an acidic amino acid in positions 2 and 5, and a tryptophan in positions 4, 7, and 8.

In another embodiment, the inhibitor of the invention is a decapeptide containing (from the N-terminus to the C-terminus) a basic amino acid in position 1, an acidic amino acid in positions 2 and 5, and a tryptophan in positions 4, 7, and 8.

The peptide or analog that can be used preferably covers the residues 389-407 of the HIV-RT, more preferably 395-404.

By peptide or analog, it is meant to understand a molecule comprising a series of amino acids, that can be natural or not, said molecule being linear or circular, and capable of being modified, by linkages such as glycosylation or branches of amino acids such as glutamylation on glutamate(s).

A peptide according to the present invention can be obtained by genetic engineering, after introduction of a DNA vector carrying a nucleic acid sequence coding for the peptide sequence into a host cell (procaryotic or eucaryotic cell, such as bacteria, yeast, mammalian cell), and producing the peptide within said host cell. Some induction systems allow the production of a large amount of peptide, and are techniques well known by the person skilled in the art.

The peptides or analogs according to the present invention can also be obtained by chemical synthesis, using one of the many known peptidic synthesis. One could cite the techniques using solid phases, total or partial, by fragment condensation, or a synthesis in classical solution.

The peptides or analogs of the invention may comprise non natural or modified amino acids. Among these non naturally occurring amino acid, one could cite without being limitative, ornithine, norleucine, norvaline, hydroxyproline, hydroxylysine, ethylglycine, ethylasparagine. A list of modified amino acids has been edited by the WIPO in the definition of the sequence norm ST 25.

The peptides or analogs according to the present invention can contain modifications of the amino acids that are found naturally or not, among them glycosylation.

The sequence of the peptides or analogs according to the present invention can also be modified, without modification of the biological activity (inhibition of HIV-RT replication, in particular through inhibition of HIV-RT dimerization), in particular to increase their solubility, preferably in aqueous solvents.

The peptides or analogs according to the invention may be modified in order to increase their stability *in vitro* and/or in *vivo*. For example, one can use D amino acids and/or block the N and/or C-termini of the peptides.

It is possible to replace some minor amino-acids to increase the stability or allow a better penetration of the peptide or analog in the cell.

It is to understand that the acceptable modifications to the peptides or analogs maintain the biological activity of said molecules, which is to inhibit HIV replication.

The process for the purification of peptides are known by the person skilled in the art. Recombinant peptides can be purified from lysates or cellular extracts, from the supernatant of the culture medium by techniques used individually or in combination.

The techniques that can be used to purify peptides prepared through a recombinant host or by chemical synthesis include fractionment, chromatography, immunoaffinity techniques, using monoclonal or polyclonal specific antibodies...

The inhibitor according to the present invention exhibits a better potency when it further comprises a vector allowing the penetration of the peptide or analog into a mammalian cell.

The person skilled in the art can design such a vector, which preferably is comprised in the group consisting of liposomes, polymeric protein-binding cations, proteins, peptides, micro- or nanoparticles.

In a preferred embodiment of the invention, the vector used to facilitate the penetration of the peptide in the inhibitor of the present invention comprises the peptide MPG (SEQ ID N° 2), the amphipatic sequence of peptide MPG (SEQ ID N° 3) or an analog thereof.

In a preferred embodiment of the invention, the vector used to facilitate the penetration of the peptide in the inhibitor of the present invention is the peptide MPG (SEQ ID N° 2), the amphipatic sequence of peptide MPG (SEQ ID N° 3) or an analog thereof.

In a preferred embodiment, the peptide and the vector in the inhibitor according to the present invention are in the form of a complex.

In another embodiment, the peptide and the vector in the inhibitor according to the present invention are linked by a covalent liaison.

In a very preferred embodiment, the inhibitor according to the invention is formed by a peptide comprising peptide p7 and peptide MPG or the amphipatic sequence of peptide MPG, or analogs thereof.

In the most preferred embodiment, the inhibitor according to the invention is peptide p7++ (retroinhibase 1, SEQ ID N° 4), or an analog thereof.

The invention is also drawn to a pharmaceutical composition comprising an inhibitor of HIV replication according to the invention, and an appropriate excipient. Said compositions are preferably formulated for administration to mammals, in particular human beings. They are preferably formulated to be administrated by oral, sublingual, subcutaneous intramuscular, intravenous, transdermal, rectal way.

The pharmaceutical composition may be a tablet, a capsule, a powder, a pill, a suppository, a solution (injectable by a method as previously cited) or a suspension.

The excipient may be gelatin, starch, lactose, arabic gum, talc, or other known pharmaceutical vehicles. The tablets may be coated by sucrose, or other appropriate compounds.

The pharmaceutical composition according to the invention may be treated as to achieve a sustained or retarded activity, or for the release of a predetermined amount of inhibitor in a continuous way.

The capsule may be obtained by mixing the inhibitor with a diluent and pouring the mixture in soft or hard capsules.

A syrup may be obtained by mixing the inhibitor with an sweetener, an antiseptic, a tasting agent, and an appropriate colorant.

Powders or granules may contain the inhibitor mixed with dispersion agents, or wetting agents, optionally with tasting agents and/or sweeteners.

For rectal administration, suppositories may be prepared with binding agents, melting at rectal temperature, such as cocoa butter or polyethyleneglycols.

For injectable administration, one could use aqueous suspensions, saline isotonic solutions or sterile solutions that contains dispersions agents, and/or wetting agents pharmacologically compatibles.

The inhibitor may also be formulated as a microcapsule, with possibly one or more additive supports.

The examples in the present application show that the inhibitors of the invention are very potent against the replication of HIV strains *in vitro*. Furthermore, they are also very potent against both HIV-1 and HIV-2 strains, as well as against drug- and multidrug-resistant strains.

Therefore, the invention is also drawn to the use of an inhibitor, or a composition according to the invention, for the manufacture of a medicament to be used in the treatment of an HIV infected patient, whether HIV is a HIV-1, HIV-2, drug sensitive, drug-resistant or multidrug-resistant HIV virus.

It is foreseen that the medicament of the present invention will be used simultaneously or in combination with one or more other anti-HIV medicament(s). Indeed, the best current clinical results for limiting HIV infections are obtained by using multiple drugs at the same time. The invention presents a new therapeutic class of molecules to be used against HIV, and shall therefore be added to the current treatment regimens.

The other anti-HIV medicaments that can be used at the same time as the medicament or the inhibitor of the invention include protease inhibitors and inhibitors of the HIV-RT, such as nucleoside or non-nucleosides inhibitors.

It is also worth noting that the inhibitors according to the present invention are directed against conserved region of the HIV-RT, that is essential for the dimerization of the protein. Therefore, by using the inhibitor of the invention, one can prevent the dimerization of the HIV-RT, which may prevent the reverse transcription of the virus RNA to DNA, and its integration within the genome.

Furthermore, the inhibitors and compositions of the invention are advantageous in that they target a conserved region of the virus genome, that is probably not very prone to mutations, as it is essential for the dimerization of the HIV-RT protein. It is therefore expected that there will be less resistant strains to the inhibitors of the invention than with other inhibitors of HIV-RT, such as nucleoside analogs.

The inhibitors of the present invention show an inhibition of virus replication for concentrations in the range of 10^{-7} or 10^{-8} M *in vitro*, that is lower than the concentration needed for AZT. Furthermore, cell toxicity is only observed for inhibitor concentration 10,000 times higher.

It is therefore foreseen that the inhibitors or the composition according to the present invention will be administered at a dose that will allow them to be effective. Such a dose is said to be therapeutically effective, i.e. anti-virally effective, without a reduced toxicity.

According to the literature that reports on the use of peptides as therapeutic agents (40), the medicament of the invention may be administered at a dose of about 1 to 1000 mg/day, or more preferably at a dose of about 20 to 700 mg/day.

The invention is also drawn to a method for treating or inhibiting an HIV infection comprising administering to a human in need thereof a therapeutically effective (anti-virally effective) amount of an inhibitor, or a composition according to the invention, optionally in combination with a therapeutically effective amount of one or more other anti-HIV medicament(s) (such as nucleosides or non-nucleosides inhibitors of the reverse transcriptase, protease inhibitors).

The method is effective against HIV-1 or HIV-2, and particularly against drug- or multidrug-resistant HIV.

EXAMPLES

Example 1: Methods

1.1 Viruses

The HIV strains used in this study were already described: HIV-1 BRU (28), HIV-2 ROD (29), HIV-1 ELI (30), HIV-2EHO (31,32), HIV-1 NDK (33), HIV-1 RF (34), HIV-1 SF2 (35), nevirapine-resistant HIV-1N119 (20), HIV-1 RTMC (21), HIV-1 RTMF (18), HIV-1 74V (36), and HIV-1 RTMDR1 (24). These viruses were propagated in CEM cells (a CD4+/CXCR4+ human T-cell line).

1.2. Cells

The CD4+, CXCR4+ lymphoblastoid CEM cell line was purchased from the American Type Culture Collection (CCRF-CEM, ATCC # CCL 119, Catalogue of cell lines and hybridomas, ATCC, Bethesda, MD). CEM cells were cultured in RPMI 1640 medium containing 1% penicillin-streptomycin (PS) antibiotic mixture, 1% glutamax (Gibco-BRL, Eragny, France) and 10% FCS (Gibco), to a density of 5 X 10^5 cells/ml in a 5% CO_2 atmosphere. The HeLa-LTR-βgal indicator cell line (37) stably transfected with CD4 and CCR5 (MAGIC-5) cells was previously described (38) , were grown in DMEM containing 1% PS, 1% glutamax, 1 mg/ml G418, and 10% FCS.

1.3. Peptides

Peptides (p7 (SEQ ID N° 1), p237 (SEQ ID N° 5), MPG (SEQ ID N° 2), p7++ (SEQ ID N° 4), and p7+ (SEQ ID N° 6)) were synthesized by solid phase peptide synthesis using aminoethyldithio-2-isobutyric acid-expensin resin with a 9050 Pepsynthetizer (Millipore, UK) according to the Fmoc(*N*-(9-fluorenyl)methoxycarbonyl)/*tert*-butyl method, purified by semi-preparative HPLC and identified by electrospray mass spectrometry and amino acid analysis. In some case, to increase their stability, the peptides were acetylated at the N

terminus and linked to a cysteamide group at the C-terminal part as previously described (14).

1.4. Formation of p7/MPG complex

Peptide p7 and MPG were mixed, and peptide p7 binds to MPG (probably the hydrophobic domain), with saturation taking place for a concentration of p7 about 20-fold lower than of MPG. From the K_d and the saturation concentration, the ration was estimated to 30 molecules of MPG for one molecule of p7. The MPG/p7 complex was further assessed as a complex of p7-MPG at a 1/20 ratio (15, incorporated herein by reference in its totality).

1.5. Infection of cells

RT activity assay. CEM cells were incubated for 30 min at 4°C with 100 µl of stock HIV preparation corresponding to 1000 x 50% tissue culture infective dose ($TCID_{50}$)/ml, then cells were washed five times and cultured at 5 x 10^5 cell/ml in 24-well microplates in the presence or absence of MPG/p7 (at 10^{-8} and 10^{-7} M) or AZT (AZT was purchased from Boehringer Mannheim, Germany) additive. Viral production was monitored twice a week by measuring reverse transcriptase activity in 1 ml of cell-free supernatant as previously described (39).

β-gal activity assay. MAGIC-5 cells expressing the β-gal reporter gene cloned downstream of the HIV-1 LTR promoter were plated in 24-well plates at 5 x 10^5 cells/ml and incubated with 50 µl of stock HIV preparation corresponding to 1000 x 50% tissue culture infective dose ($TCID_{50}$)/ml in the presence or absence of MPG/p7 (at 10^{-8} and 10^{-7} M) or AZT additive. After 3 days in culture, cells were lysed and β-gal activity was determined by incubating 200 µl of total cellular extracts for 1 h at 37°C in 1.5 ml buffer containing 80 mM NA_2HPO_4, 10 mM $MgCl_2$, 1 mM 2 ME and 6 mM o-nitrophenyl β-D-galactopyranoside (ONPG). β-gal activity was evaluated by measuring absorbance at 410 nm.

Example 2: inhibition of HIV-1 and HIV-2 isolates replication in CEM cells by MPG/p7 complex

MPG/p7 was previously shown to inhibit HIV-1BRU RT dimerization *in vitro* and HIV-1BRU replication in CEM cell culture (15, incorporated herein by reference).The susceptibility of two reference strains of laboratory-adapted HIV-1 and HIV-2 to MPG/p7 was first determined.

A representative experiment of inhibition of virus production (HIV-1BRU and HIV-2ROD) in infected CEM cells treated with MPG/p7 (10^{-6} M), a 15-mer control peptide 237

(10^{-6} M), or AZT (10^{-5} M), is shown in Figure 1. RT activity monitored in cell free culture supernatant of infected cells from day 3 to day 17 after virus exposure, indicated that MPG/p7 at concentrations above 10^{-8} M, totally inhibits HIV-1BRU replication during the 17 days of incubation whereas RT activity observed at the end of culture in samples treated at concentration of 10^{-9} M revealed a very slow virus propagation that was undetected at earlier time-points. Only a 3-days delay in HIV-1 replication was found at a concentration of 10^{-10} M of MPG/p7.

Under similar experimental conditions HIV-2ROD propagation was strongly delayed at concentrations of MPG/p7 above 10^{-7} M, whereas a 3-days delay in HIV-2 replication was observed using a concentration of 10^{-9} M of MPG/p7.

This result was confirmed by monitoring the expression of HIV-1 antigen by $P24^{gag}$ antigen capture assay on day 17 post infection (data not shown).

Example 3: inhibition of HIV-1 and HIV-2 isolates replication in MAGIC5 cells by MPG/p7 complex

The pattern of reactivity of MPG/p7 using several isolates from different clades of HIV-1 and HIV-2 isolates (see Table 1) was next determined.

The efficiency of MPG/p7 monitored using the previously described MAGIC5 transfectant cells that express surface CD4, CXCR4 and CCR5 receptors and contain a reporter gene under control of an HIV-1 promoter that can be induced upon infection of the cells. This assay was chosen because it gives a result within 72h and requires much less peptide in each experiment than classical infection assays.

All strains tested were found to be susceptible to MPG/p7 at concentrations of 10^{-8} M (Fig. 2). This included X4 and R5/X4 strains of HIV-1 from clades B and D and HIV-2 clades A and B.

Although only HIV-1 clades B and D and HIV-2 clades A and B isolates were tested, it can be assumed that the 7 isolates that were used in the present study are representative of the different sequences that can be encountered within HIV-1 clades A, B, C, D, F, G, H, O and HIV-2 clades A and B.

Indeed, comparisons of the sequence of residues 395-404 of HIV-1 BH_{10} RT (KETWETWWTE, SEQ ID N° 1) to the corresponding sequences available from the Los Alamos data base (9) (Table 1) reveals that the major substitutions observed between the

consensus B and the other clades of HIV-1 are found in one of the HIV-1 B or D, or HIV-2 viruses tested.

For example, the HIV-1 clade C (see consensus C sequence) that is most predominant in India and causes more than 70% of infections in southern Africa and 96% in northern Africa shows a $T_{400}A$ substitution also encountered in HIV-1 RF and HIV-1 NDK and an $E_{404}D$ substitution also encountered in HIV-2 EHO.

Therefore, in most cases substitutions do not alter the character of the residues, confirming that this region is highly conserved in HIVs.

These observations suggest that irrespectively of type, clade an geographic origin, all human lentiviruses containing a decapeptide containing (from the N-terminus to the C-terminus) a basic amino acid in position 1, an acidic amino acid in positions 2 and 5, and a tryptophan in positions 4, 7, and 8 at the interface of the connecting subdomains of the p66 and p51 subunits can potentially be inhibited by MPG/p7.

Example 4: crystallographic analysis of the HIV-RT

The crystallographic structure of HIV-1 RT, reveals that the residues 395-404 are involved in the p66/p51 interface contacts and are essential in the stabilisation of both the connection subdomain.

Secondary structure predictions and molecular modeling suggest that in all the isolates these residues are folded into an α-helix, as observed in the X-ray structure of HIV-1 (16,17).

In both subunits, the highly conserved Trp residues, Trp_{398}, Trp_{401} and Trp_{402} form a cluster of aromatic residues together with Tyr_{405}, Trp_{410}, and Phe_{416} which stabilizes the dimer interface by intra- and inter-subunit contacts. In p51 the hydrohobic cluster involves other contacts which maintains the conformation of the palm domain of p51 (Trp_{24}, Phe_{61}, Leu_{368}, Leu_{391}, Val_{372}) and the thumb-domain of p66 (Arg_{356}, Arg_{358}, Gln_{373}). In p66, additional contacts are made with the RNase-H domain (Val_{423}, Leu_{425}).

Analysis of the amino acid substitutions indicated that whatever the sequence mentioned in Table 1, the property of the residues is conserved in order to maintain the organization of the hydrophobic pocket and the α-helix conformation.

Substitution $K_{395}R$ or $E_{399}D$ retains the basic characteristic, essential for helix stability and interactions with Trp_{24}, Phe_{416} and Trp_{414}.

Substitution $E_{396}D$ keep the acidic property essential for the interaction with residues Gln_{394} in p51 and the two Arg_{356} and Arg_{358} in p66.

Thr_{397} is conserved in all of the concensus, excepted in HIV-2 concensus A, in both subunits this residue is surrounded by hydrophobic residues, which cannot be altered when replaced by isoleucine or valine.

Thr_{400} is one of the most variable residue of this motif, therefore none of the substitution alters the organization of the aromatic cluster.

Moreover, the substitution $T_{400}Q$ observed in all HIV-2 consensus increases interaction between p51 and p66 subunits and may explain the higher stability of HIV-2 RT[11].

Taken together, these data indicate that MPG/p7 inhibits a wide range of HIVs. Based on these results we conclude that the integrity of the aromatic cluster which is essential for dimer formation as well as for the structural integrity of both subunits, is conserved in all of the isolates described in Table 1. This explains why a drug like p7, which targets the aromatic cluster prevents the dimer formation of all of these isolates.

Example 5: Susceptibility of anti-RT drug resistant HIV-1 strains to MPG/p7

The phenotypic identification of drug-resistant HIV-1 emerging during unsuccessful antiretroviral therapy has enable the definition of drug resistant genotypes of HIV-1.

Several mutations in RT are consistently in association with resistance to one or more anti-RT drugs. The growing number of reports documenting mutations which confer resistance to both nucleoside and non-nucleoside RT inhibitors indicates that one of the first selection criteria that a new antiviral compound targeting RT should meet, is its capacity to inhibit anti-RT drug resistant HIV-1 strains.

According to the compilation of mutations in HIV RT published by the Los Alamos National Laboratory (9), there are at least 45 amino acid residues in RT for which mutations result in a significant change in the virus susceptibility to one or more anti-RT drugs.

Interestingly, and to the best of our knowledge, there is actually no mutation reported affecting the residues of HIV-1 RT that are target for MPG/p7. Moreover, that mutation of Trp_{398} and Trp_{410} (HIV-1 BH10) strongly affected the stability of the dimeric form of HIV-1 RT *in vitro* (Morris and Divita, unpublished data), strengthens the hypothesis that mutations occurring in this region may affect RT dimerization thereby disabling viral replication.

To determine whether mutant HIV-1s that resist to both nucleoside and/or non-nucleoside RT inhibitors are sensitive to MPG/p7, five reference strains of anti-RT drug resistant HIV-1 were assayed for susceptibility to MPG/p7.

Table 2 summarizes the characteristics of RT drug resistant phenotype of these viruses, namely HIV-1 RTMF, HIV-1 RTMC, HIV-1 74V, HIV-1 N119 and HIV-1 RTMDR1, and the type of mutation conferring these phenotypes.

The different escape mutant viruses studied turned to be sensitive to MPG/p7 treatment (Fig. 3). It is however worth noting that the concentration of MPG/p7 required to inhibit HIV-1 RTMC and HIV-1 RTMDR1 (Fig. 3*c* and *f*) was higher than that required to block the other viruses. For example, 10^{-6} M of MPG/p7 was required for completely inhibit HIV-1 RTMC (data not shown).

The fact that the sensitivity to zidovudine of HIV-1 RTMC and HIV-1 RTMF escape mutant strains was very similar in the present experiment and in the experiment previously reported by Larder and co-workers (18), validated the observations relatively to MPG/p7 effect on the different escape mutant viruses.

Example 6: inhibition of HIV replication by peptide p7++

In order to further study the properties of peptide p7, chimeric peptides having the transmembranaire transport properties of MPG (amhipathic sequence) and anti HIV-RT properties of p7 were designed and synthesized.

Peptide p7++ and p7+ were used on MAGIC-5 cells, infected with the ROD (HIV-2) and BRU (HIV-1) strains.

Figure 4 shows that peptide p7++ (retroinhibase 1) exhibits the same activity than the MPG-p7 complex at about the same concentration.

The use of peptide p7++ on cells infected by drug-resistant viruses demonstrates that this peptide is also capable to inhibit the replication of such strains (figure 4).

Table 1 Amino acid alignment with the p7 sequence of BH10 strain

Type	Strain	Clade	Country of origin	Sequence
HIV-1				395 404
	BH10			KETWETWWTE (p7)
	BRU	B	France	KETWETWWTE
	RF	B	Haiti/USA	KETWE**A**WWTE
	SF2	B	USA	KETWE**A**WW**M**E
	NDK	D	Zaire	KETWETWW**I**E
	ELI	D	Zaire	KETWETWW**A**E
HIV-2				
	ROD	A	Cape Verde	**R**E**I**WE**Q**WW**D**N
	EHO	B	Ivory Coast	**R**ETW**DQ**WWT**D**
HIV-1				395 (p7) 404
				KETWETWWTE
Consensus		A		KETWE$_{T/A}$WWT$_{E/D}$
Consensus		B		KETWE$_{T/A}$WW**M**E
Consensus		C		KETWE**A**WWT**D**
Consensus		D		KETWE$_{T/A}$WW**X**$_{E/D}$ (**X**=T$_{/A/V/I}$)
Consensus		F		KETW**D**TWWTE
Consensus		G		KETWE**V**WWTE
Consensus		H		KETWETWWTE
Consensus		0		**R**ETWETWW**AD**
HIV-2				
Consensus		A		**R**$_{E/D}$**Z**WE**Q**WW**D**$_{N/D}$ (**Z**=T$_{/V/I}$)
Consensus		B		**R**ETW**DQ**WWT**D**

Sequences are from ref.9

Table 2 Main characteristics of anti-RT drug resistant HIV-1 isolates

Strain	RT Genotyping	Phenotype
HIV-1 RTMF	215Y	AZT-resistant
HIV-1 RTMC	67N, 70R, 215F, 219Q	AZT-resistant
HIV-1 74V	74V	resistant to ddI and ddC
HIV-1 N119	181C	resistant to nevapirine and non nucleoside RT inhibitors
HIV-1 RTMDR1	41L, 74V, 106A, 215Y	resistant to AZT, ddI, nevapirine, non nucleoside RT inhibitors

Viruses phenotype and reverse transcriptase genotype are adapted from refs. 18, 20, 21, 23, and 36. HIV-1 RTMF, RTMC, 74V, RTMDR1, and N119 viruses utilize CXCR4 (some isolates are R5X4 dual tropic strains).

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